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Physiological regulation of pod set in soybean (*Glycine max*
L. Merr.) during drought at early reproductive stages

Ph.D. Dissertation by

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This thesis is submitted together with the enclosed four articles in partial fulfilment of the requirement of the Ph.D. degree at The Royal Veterinary and Agricultural University (KVL), Copenhagen, Denmark

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Table of content

ACKNOWLEDGEMENTS	I
TABLE OF CONTENT	II
RESUMÉ	IV
SUMMARY	VII
LIST OF ORIGINAL PUBLICATIONS	X
1. GENERAL INTRODUCTION.....	1
1.1. RATIONALE	1
1.2. OBJECTIVES.....	2
2. GENERAL LITERATURE REVIEW	3
2.1. THE CROP – SOYBEAN.....	3
2.1.1. <i>Crop origin and cultivation history</i>	3
2.1.2. <i>Botanical characteristics</i>	3
2.1.3. <i>Growth and development</i>	4
2.1.4. <i>A flower and pod staging system around anthesis</i>	7
2.1.5. <i>Plant water use</i>	9
2.2. YIELD DETERMINATION IN SOYBEAN	10
2.2.1. <i>Yield components</i>	10
2.2.2. <i>Effect of anthesis-drought stress on pod set</i>	10
2.3. REGULATION OF CROP REPRODUCTIVE ABORTION UNDER ANTHESIS-DROUGHT STRESS.....	11
2.3.1. <i>Carbohydrate supply – source activity</i>	12
2.3.2. <i>Carbohydrate utilization – sink capacity</i>	13
2.3.3. <i>Phytohormones</i>	16
2.3.4. <i>Long-distance signals</i>	19
3. A BRIEF SUMMARY OF THE EXPERIMENTAL STUDIES IN SOYBEAN ..	24
3.1. MATERIALS AND METHODS	24
3.2. RESULTS AND DISCUSSION	25

3.2.1.	<i>The critical stage of pod abortion</i>	25
3.2.2.	<i>Source and sink limitations and their relationships to pod set</i>	25
3.2.3.	<i>Roles of ABA and exogenous CKs in the regulation of pod set.....</i>	29
3.3.	CONCLUSIONS	32
3.4.	PERSPECTIVES	32
4.	REFERENCE.....	34

RESUMÉ

Vandmangel er en af de faktorer, der i størst omfang begrænser afgrøde-produktiviteten på verdensplan. Udbyttet af sojabønne (*Glycine max* L. Merr.), der er en vigtig olieafgrøde, bliver meget påvirket af tørkestress, specielt når stressen foregår i blomstringsperioden og under bælg-udviklingen. Udbyttetabet skyldes hovedsagelig et tiltagende bælgtab, hvilket resulterer i færre antal frø per arealenhed. Men indtil videre er man ikke klar over, hvad det er for nogle mekanismer, der forårsager bælgtabet. Hovedformålet med dette studium var at undersøge den fysiologiske basis for bælgtab i sojabønner, som udsættes for tørkestress under det tidlige reproduktive stadie.

Tidlig bælguvikling i sojabønner er karakteristisk ved en tidlig celledeling i de unge frøanlæg og er præget af en hurtig bælguvikling; begge processer er meget følsomme overfor tørkestress. Tørkeprovokeret kulhydrattab og forandringer i planternes koncentration af endogen abscisinsyre (ABA) kan have væsentlig effekt på bælgvækst og -udvikling, og kan således være medvirkende årsag til bælgtab. For at teste disse hypoteser blev der gennemført 4 kar-forsøg med sojabønner (cv. Holladay) i et klimakontrolleret drivhus i løbet af 2002 og 2003. Mængden af transpirationsbart jordvand i karrene (FTSW) blev brugt som et mål for jordvandstatus. De to første forsøg blev udført for at undersøge det kritiske punkt for bælgtab og de dermed forbundne ændringer i de biofysiske og biokemiske faktorer, nemlig, plante-vand-relationer, fotosyntese, endogent ABA-niveau og kulhydrat koncentrationer under tørkestress. Det tredje forsøg blev udført for at undersøge effekten af tørkestress på koncentrationen af ABA i bælgene og for yderligere at studere forholdet mellem bælgdannelse i forhold til de ovennævnte faktorer på det kritiske, følsomme tidspunkt for bælgtab. Til slut, i det sidste forsøg, blev der gennemført manipulations-studier for at efterprøve de postulater, der var fremsat under de tidligere forsøg.

Resultaterne viste, at hård tørkestress reducerede bælgsætningen væsentligt til 40%, og det kritiske tidspunkt for bælgtab var 3-5 dage efter anthesis (DAA), hvor celledelingen var aktiv i frugtknuderne. Tørke på et senere tidspunkt, hvor bælgyldningen var begyndt, reducerede frøstørrelsen, men havde ingen væsentlig effekt på bælgsætningen. Bælgyvandpotentialet formindskedes under tørke, imidlertid blev bælgsaftspændingen holdt på et lignende niveau som hos de fuldt vandede kontrolkar. ABA koncentrationen forøgedes væsentligt i xylemsaften, bladene og bælgene i tørkestressede planter. Xylem

ABA og blad ABA var tilsyneladende kilden til, at ABA akkumuleredes i de tørke-stressede bælg.

Kulhydrat stofskiftet blev afbrudt af tørkestress i både blad- og blomsterorganer. I bladene formindskede tørkestress fotosyntesehastigheden, stivelse- og sucrosekoncentrationer, men forøgede koncentrationerne af hexoser (glucose + fructose), hvilket indikerer en source begrænsning. I blomster og bælg forøgede tørkestress sucrose- og hexosekoncentrationerne, men formindskede koncentrationen af stivelse, opløselig invertase aktivitet, og hexose/sucrose forholdet, hvilket indikerer, at bælgens evne til at bruge den indgående sucrose blev forringet af tørkestress. Som en konsekvens af både source og sink-restriktioner, blev ikke-struktureret kulhydrat (sucrose + hexose + stivelse), som var akkumuleret i bælgene, væsentligt reduceret under tørkestress.

Jordvandstærsklen (FTSW) for reduktion i bælgvækst og bælgsætning var henholdsvis 0.43 og 0.30. Bælgvæksten blev reduceret før en væsentlig sænkning af bælgvands-potentialet blev påvist, og nedgangen i frisk bælgvægt var i god overensstemmelse med den tiltagende ABA koncentration i xylemsaften, der indebar at rodsignalet, og ikke bælgvands-potentialet, kontrollerede bælgvæksten under jordudtørringen. Bælgsætningen begyndte først at formindskes, når bælgvands potentiale var aftaget og bladfotosyntese hastigheden og frisk vægt af bælg var faldet med henholdsvis 40% og 30%, og bælg ABA koncentrationen var forøget 1.5 gange sammenlignet med de fuldt vandede kontrolplanter. Under tærsklen for vandpotentialet fortsatte bælgsætningen med at aftage og var positivt korreleret med fotosyntesehastigheden og friskvægten af bælg, mens sætningen var negativt korreleret med ABA koncentration i bælgene.

Manipulationsforsøg viste, at anvendelse af 0.1 mM ABA på planten formindskede gasudvekslingen og bælgsætningen i fuldt vandede sojabønner. I tørkestressede planter provokerede ABA behandling stomatalukning på et tidligt tidspunkt i jordudtørringen, hvilket førte til et højere bladvandspotentiale, som opretholdt større gasudveksling, resulterende i en forøget bælgsætning sammenlignet med planter uden anvendelse af ABA. Ved at "spraye" 1 mM 6-benzylaminopurine (BA, en kunstig cytokinin) på toppen af afgrøden forøgedes gasudvekslingen og bælgsætningen i fuldt vandede planter, men det formindskede bladvandspotentialet, gasudvekslingen og i lille omfang bælgsætningen i de tørkestressede planter. I ABA- og BA-behandlede planter var bælgsætningen lineært

relateret til blad fotosyntesen, hvilket medførte at de to hormoner influerer bælg-sætningen delvis ved at begrænse fotosynteseprodukt tilgængeligheden.

Baseret på de ovenfor anførte resultater blev det konkluderet at:

- Tidlig bælgudvikling (3-5 DAA) er det mest sensitive stade for bælgtab i sojabønner, der udsættes for tørkestress.
- Tilstrækkelig med fotosynteseprodukt forsyning er livsvigtig for bælg-sætningen. Tørke-inducerede reduktioner i aktuel- og reservekulhydrat forsyninger bidrager til bælgtab under den kritiske, abortfølsomme fase i bælgudviklingen.
- Bælgvæksten er mere sensitiv overfor underskud af jordvand end bælg-sætning er. Xylemsaft med ABA fra roden kontrollerer tilsyneladende bælgvæksten, hvorimod lavt bælgvandspotentiale afbryder kulhydrat metabolisme i bælgene og provokerer bælgtab.
- ABA og BA spiller vigtige roller i kontrollen af bælg-sætningen i sojabønner. De to hormoner udøver deres effekt på bælg-sætning direkte ved at påvirke bælg-dannelsesvævets metabolske processer (f.eks. celledeling) eller indirekte gennem påvirkning af stomatal ledningsevne og således fotosyntese produktforsyningen.

Nøgleord: abscisinsyre, kulhydrater, 6-benzylaminopurine, tørkestress, *Glycine max* L., bælgtab, bælgvækst

Summary

Water scarcity is a major factor limiting crop productivity worldwide. Yield in soybean (*Glycine max* L. Merr.), a staple oilseed crop, is highly affected by drought stress, particularly when the stress occurring during flowering and early pod expansion. The yield loss is due mainly to an increased rate of pod abortion resulting in a less number of seeds per unit area. However, until now the underlying mechanisms causing pod abortion are still poorly understood. The overall objective of this study was to investigate the physiological basis for pod abortion of soybean exposed to drought stress during early reproductive development.

Early pod development of soybean is characterised by active cell division in the young ovules and is marked by rapid pod expansion; both processes are very sensitive to drought stress. Drought-induced carbohydrate deprivation and change in the concentration of endogenous abscisic acid (ABA) of the plants could have significant effects on pod growth and development, and may thus be involved in inducing pod abortion. To test these hypotheses, four pot experiments with soybean (cv. Holladay) were conducted in an environmentally-controlled glasshouse during 2002 and 2003. The fraction of transpirable soil water (FTSW) was used as a measure of the soil water status in the pots. The first two experiments were designed to investigate the critical stage for pod abortion and the associated changes of biophysical and biochemical factors, viz. plant water relation characteristics, photosynthesis, endogenous ABA and carbohydrate concentrations under drought stress. The third experiment was set up to investigate the effect of drought stress on pod ABA concentration and to further study the relationships of pod set to the aforementioned factors at the critical, abortion-sensitive stage. Finally, in the last experiment, manipulation studies were carried out in order to verify the postulations formulated during the former experiments.

The results showed that severe drought stress significantly decreased pod set up to 40% and the critical stage for pod abortion was 3-5 days after anthesis (DAA) when cell division was active in the ovaries. Drought at later stages when pod filling had begun reduced seed size but had no significant effect on pod set. Pod water potential decreased by drought, however pod turgor was maintained at similar level to the well-watered controls. ABA concentration increased significantly in the xylem sap, leaves, and pods of drought-

stressed plants. Xylem-borne ABA and leaf ABA were seemingly the source of ABA accumulated in the drought-stressed pods.

Carbohydrate metabolism was disrupted by drought stress in both leaves and floral organs. In leaves, drought stress decreased photosynthetic rate, starch and sucrose concentrations but increased hexoses (glucose + fructose) concentrations indicating a source limitation. In flowers and pods, drought stress increased sucrose and hexoses concentrations but decreased starch concentration, soluble invertase activity, and hexoses to sucrose ratio indicating that the capacity of the pods to utilise the incoming sucrose was impaired by drought stress. As a consequence of both source and sink restrictions, non-structural carbohydrate (sucrose + hexoses + starch) accumulated in the pods was significantly reduced under drought stress.

The soil water thresholds for reduction in pod growth and pod set were 0.43 and 0.30 of FTSW, respectively. Pod growth was reduced before a significant decrease of pod water potential was detected, and the decrease of pod fresh weight was closely correlated with increasing xylem sap ABA concentration, implying that root signal and not pod water potential controlled pod growth during soil drying. Pod set began to decrease only when pod water potential had decreased and photosynthetic rate and pod fresh weight had decreased by 40% and 30% respectively, pod ABA concentration had increased 1.5-fold compared to the well-watered controls. Below the threshold water potential, pod set decreased further and correlated positively with photosynthetic rate and pod fresh weight, whilst it correlated negatively with pod ABA concentration.

Manipulation studies showed that application of 0.1 mM ABA on the canopy decreased gas exchange rates and pod set in well-watered soybeans. In drought-stressed plants, ABA treatment induced stomatal closure during early stage of soil drying leading to higher leaf water potential which maintained greater gas exchange rates resulting in an increased pod set compared to the plants without ABA application. Application of 1 mM 6-benzylaminopurine (BA, an artificial cytokinin) on the canopy increased gas exchange rates and pod set in well-watered plants, but decreased leaf water potential, gas exchange rates and slightly decreased pod set in drought-stressed plants. In ABA- and BA-treated plants, pod set was linearly correlated with the leaf photosynthetic rate implying that the two hormones exert their roles in altering pod set partly by modifying photosynthate availability.

Based on the above results, it was concluded that:

- Early pod expansion (3-5 DAA) is the most sensitive stage for pod abortion in soybean exposed to drought stress.
- Sufficient photosynthate supply is vital for setting of pods. Drought-induced reductions of current and reserve carbohydrate supplies contribute to pod abortion during the critical, abortion-sensitive phase of pod development.
- Expansion growth of pods is more sensitive to soil water deficits than pod set. Root-originated xylem sap ABA seemingly controls pod growth, whereas low pod water potentials disrupt carbohydrate metabolism in the pods and induce pod abortion.
- ABA and BA play important roles in controlling pod set in soybeans. The two hormones exert their effect on pod set directly via affecting the pod meristem metabolic processes (e.g. cell division) or indirectly through influencing stomatal conductance and thus the rate of photosynthate supply.

Key words: abscisic acid, carbohydrates, 6-benzylaminopurine, drought stress, *Glycine max* L., pod abortion, pod growth

List of original publications

- I. Liu, F., Andersen, M.N., Jensen, C.R. 2003. Loss of pod set caused by drought stress is associated with water status and ABA content of reproductive structures in soybean. *Functional Plant Biology* 30, 271–280.
- II. Liu, F., Jensen, C.R., Andersen, M.N. 2004. Drought stress effect on carbohydrate concentration in soybean leaves and pods during early reproductive development: its implication in altering pod set. *Field Crops Research*, 86, 1–13.
- III. Liu, F., Andersen, M.N., Jensen, C.R. 2004. Root signal controls pod growth in drought-stressed soybean during the critical, abortion-sensitive phase of pod development. *Field Crops Research* 85, 159–166.
- IV. Liu, F., Jensen, C.R., Andersen, M.N. 2004. Pod set related to photosynthetic rate and endogenous ABA in soybeans subjected to different water regimes and exogenous ABA and BA at early reproductive stages. *Annals of Botany* 94, 405–411.

In the following the papers will be referred to by their Roman numerals.

The four articles mentioned above aren't available in this PDF, due to restrictions from the publishers of the journals in which they are published. Instead, the articles can be found at the following pages:

I: <http://dx.doi.org/10.1071/FP02185>

II: [http://dx.doi.org/10.1016/S0378-4290\(03\)00165-5](http://dx.doi.org/10.1016/S0378-4290(03)00165-5)

III: [http://dx.doi.org/10.1016/S0378-4290\(03\)00164-3](http://dx.doi.org/10.1016/S0378-4290(03)00164-3)

IV: <http://dx.doi.org/10.1093/aob/mch157>

The full references are listed at page X

1. General introduction

1.1. Rationale

In recent years, most climate-change scenarios indicate an increase in aridity in many regions of the world (Petit et al., 1999). Drought has been the major environmental constraint to plant survival and to crop productivity (Boyer, 1982). The biggest consumer of water is agriculture, which accounts for around 70% of all freshwater withdrawals worldwide. With a growing world population and food demand, agriculture will face more competition from industrial and domestic water users. As a result, agriculture will have to use water more efficiently. This recalls for a deeper understanding about the physiological mechanisms of crop plants response to soil water deficits (Turner, 1997).

Soybean (*Glycine max* L.) is the most important grain legume crop of the world in terms of its use in human foods and livestock feeds (Fageria, 1997). Since 1970, soybean production has been at least double that of any other oilseed crop. The contribution of soybean to world oilseed production increased from 32% in 1965 to over 56% in 2000 (Soy Stats, 2001). Besides, soybeans represented over 60% of the world protein meal consumption in the 1990s (Soy Stats, 1997). In addition, soybean has been considered to be one of the most promising crops for producing bioenergy (biodiesel) in the near future (Soy Stats, 2001).

Soybean has a fairly wide range of adaptation involving a wide array of climatic, soil, and growth conditions though it is mostly grown on rain-fed land (Fageria, 1997). Soybean yield is highly affected by soil water availability (Ashley and Ethridge, 1978; Korte et al., 1983b). Drought stress at any stage of soybean development can reduce yield, but the extent and nature of damage, the capacity for recovery, and the impact on yield depend on the timing of a stress episode (Brevedan and Egli, 2003). Accumulated evidence indicates that soybean yield is more sensitive to drought stress during the early reproductive stage (i.e. flowering to early pod expansion) than other developmental stages (Boyer, 1983; Westgate and Peterson, 1993). Drought stress occurring during this period increases the rate of pod abortion (Westgate and Peterson, 1993), leads to a less number of pods per plant (Desclaux et al., 2000), and ultimately decreases seed yield (Kokubun et al., 2001).

Over the last two decades, intensive studies have focused on illustrating the physiological and biochemical basis for the failure of reproductive development of grain crops under drought stress (Westgate and Boyer, 1985, 1986; Schussler and Westgate, 1991, 1995;

Zinselmeier et al., 1995a, b; Westgate et al., 1996; Zinselmeier et al., 1999; Setter et al., 2001). Based on these studies, Saini and Westgate (2000) pointed out that reproductive abortion caused by anthesis-drought in grain crops is controlled hormonally or by the availability of carbohydrate. A large body of evidence suggests that drought induced carbohydrate deprivation, increase in endogenous ABA concentration, and an impaired ability to utilise the incoming sucrose by the reproductive sinks are potential factors contributing to seed abortion in grain crops (Setter et al., 2001). However, experimental support for these propositions comes from species other than soybeans. Until now only a few studies have attempted to explore the physiological mechanisms causing pod abortion in soybeans during drought at early reproductive stages (Westgate and Peterson, 1993; Kokubun et al., 2001). These studies only reported the data on growth and water relation characteristics of the developing pods, no information is available about the effect of soil water deficits on biochemical changes such as the concentration of endogenous ABA and carbohydrate metabolism within the reproductive structures.

1.2. Objectives

Considering that most of the world's land is classified as semiarid and that water shortage is becoming severe, a better understanding about the physiological regulation of pod abortion caused by drought stress in soybeans is therefore of primary importance for formulating management strategies to mitigate yield loss. The overall aim of this study was to investigate physiological factors that may be involved in regulating pod set in soybeans during drought at early reproductive stages. Special attention was paid on photosynthate supply from the leaves to the pods, carbohydrate metabolism in the leaves and the pods, endogenous ABA concentrations in the pods, and the possible interactions among these factors.

2. General literature review

2.1. The crop – soybean

2.1.1. Crop origin and cultivation history

The first written record of soybean cultivation is from China by Emperor Shen-Nong in 2838 B.C. Since then, soybean has been repeatedly mentioned in later records as a most important cultivated legume crop throughout Southeast Asia and particularly in China. Chinese farmers referred to a group of five sacred grains as “Wu-Gu”, which include soybean, rice, wheat, barley, and millet and were considered essential for the existence of Chinese civilisation. Soybean was introduced in Europe in 1712 and in America in the early 1800’s (Whigham, 1983). At present, the major producers of soybean are the U.S.A., China, North and South Korea, Argentina and Brazil (Whigham, 2000).

2.1.2. Botanical characteristics

Soybean belongs to the family Leguminosea, subfamily Papilionoideae, and the genus *Glycine* L. The cultivated species is *Glycine max* (L.) (Fageria et al., 1997). It is an erect, bushy herbaceous annual that can reach a height of 1.5 meters. The primary leaves of soybean are unifoliate, opposite and ovate, the secondary leaves are trifoliate. The nodulated root system consists of a taproot from which emerges a lateral root system. The plants of most varieties are covered with fine trichomes, but glabrous types also exist. The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals (one banner, two wings and two keels), one pistil and nine fused stamens with a single separate posterior stamen (Fig. 1). The stamens form a ring at the base of the stigma and elongate one day before pollination, at which time the elevated anthers form a ring around the stigma. The pod is straight or slightly curved, varies in length from two to seven centimetres, and consists of two halves of a single carpel, which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary amongst cultivars from almost spherical to elongate and flattened.

Three types of growth habit can be found amongst soybean cultivars: determinate, semi-determinate and indeterminate (Bernard and Weiss, 1973). Determinate growth is characterized by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both axillary’s and terminal racemes. Indeterminate genotypes continue

vegetative activity throughout the flowering period. Semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period.

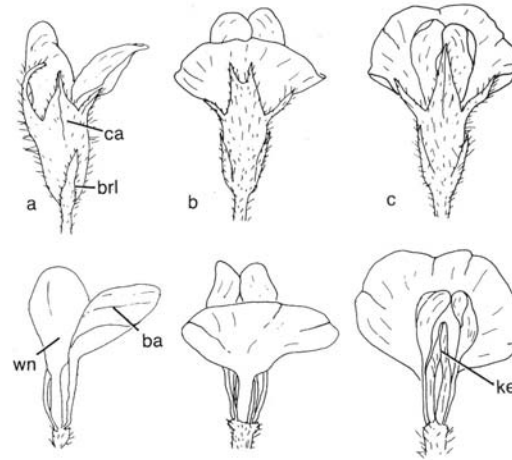


Fig. 1. Mature soybean flower. (a) Side view shows calyx with bracteoles, banner, and wing petals. (b) Adaxial or top view showing four calyx lobes, both bracteoles, banner, and wing petals. (c) Abaxial or bottom view. Three calyx lobes are visible. ba–banner petal, brl–bracteole, ca–calyx, ke–keel petals, wn–wing petal. Adapted from Carlson and Lersten (1987).

2.1.3. Growth and development

Soybean germination is epigeal, and under favourable environmental conditions, seedling begins to emerge in 4 or 5 days after sowing. In epigeal germination, the hypocotyl is active and pulls the cotyledons above ground during its growth (Nelson and Larson, 1984) (Fig. 2).

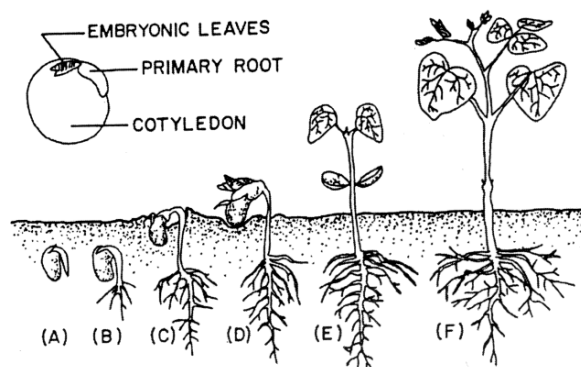


Fig. 2. Germination and seedling development of soybean. Emergence of the radicle to form the primary root (A), development of secondary roots (B), elongation of the active hypocotyl with the hypocotyl arch penetration through the soil surface (C), seedling becomes erect (D), with cotyledons attached to the first node (E), prior to drying and falling from the autotrophic seedling (F). Adapted from Nelson and Larson (1984).

Vegetative development of soybean begins with the emergence of the young seedling from the soil surface and ends with the start of flowering. Vegetative stages are designated by the number of nodes on the main stem, beginning with the unifoliate node that has a completely unrolled leaf (Fig. 3, Table 1).

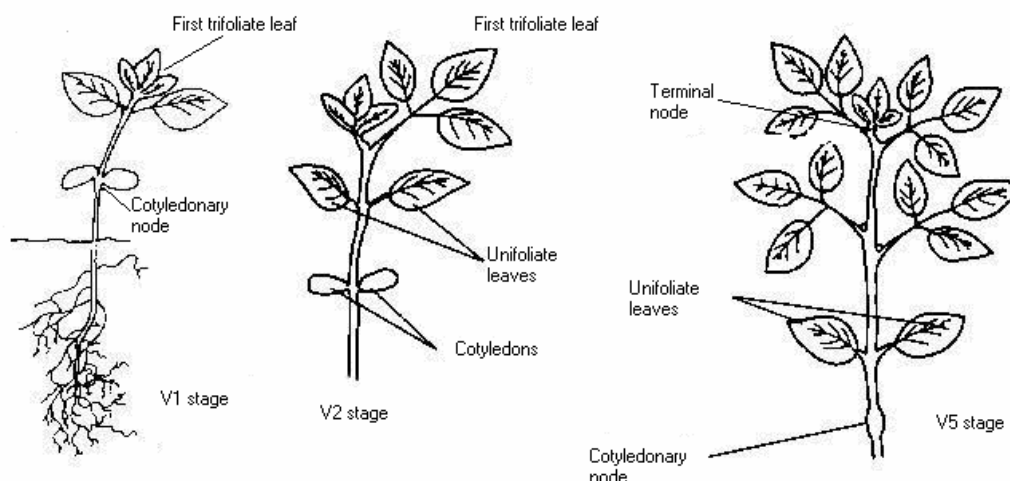


Fig. 3. Vegetative growth stages of soybean. Detailed description see Table 1.

Table 1. Description of vegetative (V) and reproductive (R) growth stages in soybean

Growth stages	Description
V1	Completely unrolled leaf at the unifoliate node
V2	Completely unrolled leaf at the first node above the unifoliate node
V3	Three nodes on the main stem beginning with the unifoliate node
V(N)	N nodes on the main stem beginning with the unifoliate node
R1	One flower at any node
R2	Flower at node immediately below the uppermost node with a completely unrolled leaf
R3	Pod 0.5 cm long at one of the four uppermost nodes with a completely unrolled leaf
R4	Pod 2 cm long at one of the four uppermost nodes with a completely unrolled leaf
R5	Seeds beginning to develop at one of the four uppermost nodes with a completely unrolled leaf
R6	Pod containing full-size green seeds at one of the four uppermost nodes with a completely unrolled leaf
R7	Pod yellowing; 50% of leaves yellow. Physiological maturity
R8	95% of pods brown. Harvest maturity

Adapted from Fehr et al. (1971).

The reproductive growth period is usually represented by flowering, pod and seed development (Fig. 4). Flowering may become visible at 25 days or may be delayed until 50 days when certain genotypes and environments interact (Whigham, 1983). The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation and cross-pollination is usually less than one percent (Abernethy et al., 1977). Flowering may occur over 4-6 weeks, depending on the environment and cultivar. After fertilisation of the flower, the pods develop slowly for the first few days, then the rate of development increases until the pod reaches maximum length after 15-20 days (Whigham, 1983).

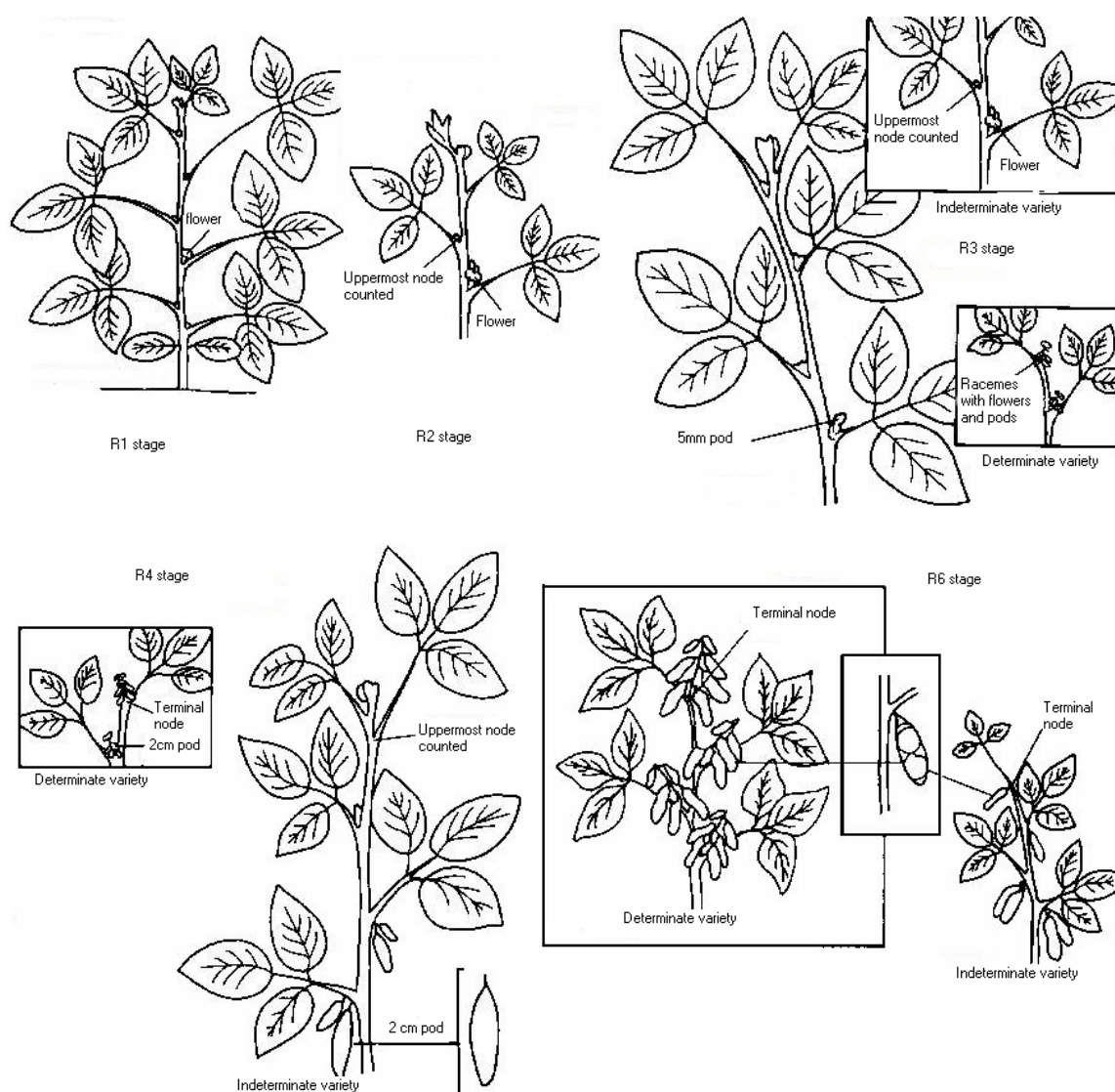


Fig. 4. Reproductive growth stages of soybean. Detailed description see Table 1.

2.1.4. A flower and pod staging system around anthesis

Peterson et al. (1992) proposed a flower and pod staging system for soybean based on the morphological characteristics of the flower a few days prior to and following anthesis (Fig. 5, Table 2). Studies of Westgate and Peterson (1993) have shown that this system is useful for categorising morphological changes resulting from drought stress and for quantifying the impact of drought stress on pod set.

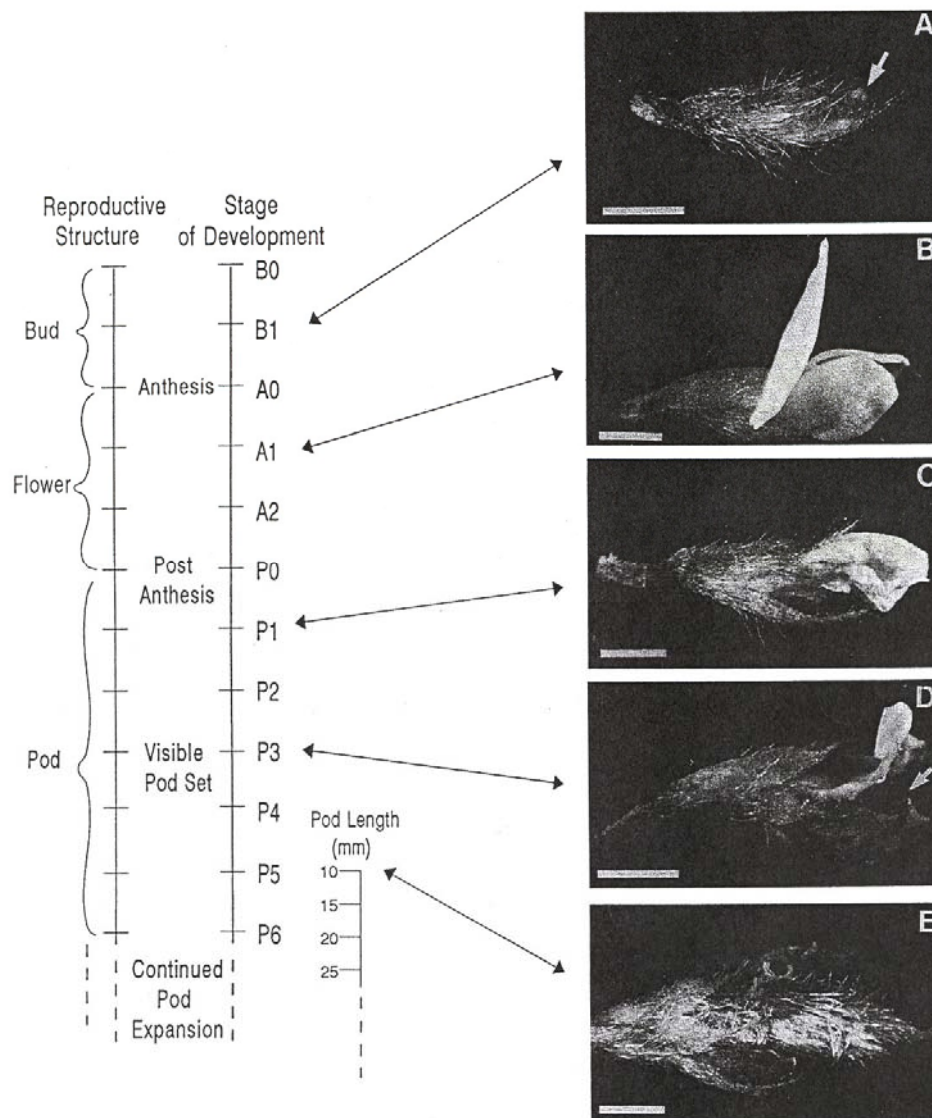


Fig. 5. Stages of soybean reproductive development from flower bud to early pod expansion. Detailed description for each stage is shown in Table 2. Adapted from Westgate and Peterson (1993).

Table 2. A flower and pod staging system for soybean

Stage	DAA	Morphological description		
		Flower	Pistil or pod	Ovules
B0	–2	No visible corolla	Pre-pollination	Maturing embryo sac
B1	–2	Corolla visible, but not fully extended beyond calyx lobes	Pre-pollination	Mature embryo sac. Central cell filled with multi-grained amyloplasts
B2	0	Banner petal fully extended	Early pollination and pollen germination	Mature embryo sac, degenerate synergid in some ovules
A0	0	Partial opening of banner petal	Pollination completed	Fertilisation beginning
A1	0	Banner petal completely reflexed, full anthesis	Pollen tube growth	Fertilisation completed; zygote formation
A2	+1	Banner petal collapsed; appears ‘hooded’	—	Zygote to 2-celled proembryos; starch grains disappearing in central cell
P0	+2	Margins of banner petal slightly wilted and rolled inward. Small brownish spots visible on banner and /or keel petals	—	2- to 4-celled proembryos; acellular endosperm
P1	+2-3	More spots, discoloration and wilting along petal edges; parts of petals withered	—	4- to 16-celled proembryos; parietal free nuclei in central cell
P2	+3-4	Completely withered petals, no turgid petal tissues visible	Pistil elongation and extension; early pod set	16-celled proembryos; acellular endosperm
P3	+4-5	Completely withered corolla	Stigma of pistil visible beyond corolla. Pod set	Up to 32-celled proembryos; ecellular endosperm
P4	+4-6	Withered corolla may be torn away or abscised from receptacle by expanding pod	Visible pod swelling and extension: 7-10 mm in length	Globular embryos with early suspensors, cellularization of endosperm beginning
P5	+5-8	Withered corolla attached at base of pod or shed	Pod 1-2 cm in length	Embryos with developed suspensors surrounded by cellular endosperm

Adapted from Peterson et al. (1992).

2.1.5. Plant water use

The water requirements of soybean vary with soil, climatic conditions, growth duration, and yield level of cultivars. Water use for soybeans can vary from 450 to 825 mm where the growing season ranges from 100 days at low altitude up to 190 days in higher altitudes (Doorenbos and Pruitt, 1977). Generally, soybean water use is low during the germination and seedling stages; the water use is especially high during the reproductive stages (R1-R6) and less during the maturation stages (Fig.6). In parallel to this pattern of water use, irrigation for soybeans was often carried out when the soil water depletion reaches 80% in the vegetative stage, 45% in early to peak flowering, 30% in late flowering to early pod development, and 80% in late pod to maturity. General field observation of drought stressed soybeans indicates that the amount of soil water available to the plants throughout all developmental stages exerts a major influence on plant growth. According to Brady et al. (1974), the best yield and most efficient water use are generally obtained when the available soil water in the root zone is not depleted by more than 50-60%. Thus, sufficient water supply, especially during the early reproductive stages is essential for soybean production under water-limited conditions.

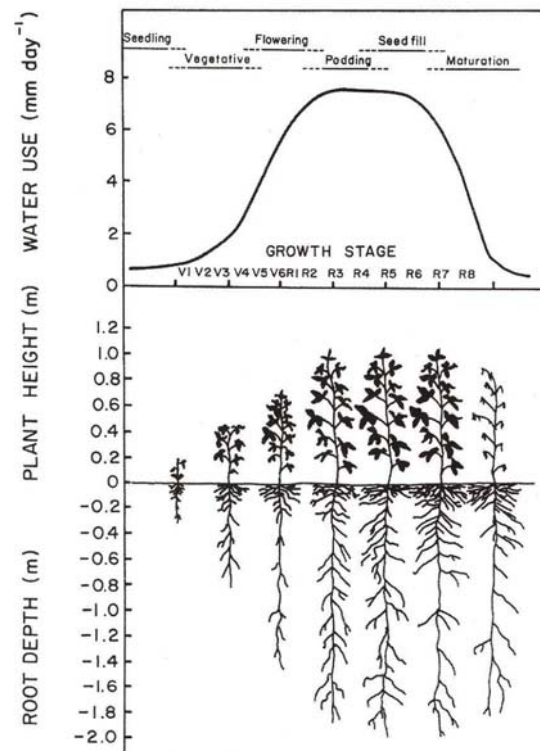


Fig. 6. Water use of soybean during vegetative and reproductive development. Modified from Van Doren and Reicosky (1987).

2.2. Yield determination in soybean

2.2.1. *Yield components*

The yield of soybean is determined by two components: the number of seeds per unit area and individual seed weight (Kokubun et al., 2001). Because seed per pod is genetically influenced (Shibles et al., 1975), the number of seeds is determined predominately by the number of pods, which is largely dependent on the number of floral buds that initiate pods and attain maturity (Desclaux et al., 2000). Soybean plants produce an abundance of floral buds, but a large proportion of the ovaries are aborted prior to developing into mature pods (Wiebold et al., 1981; Dybing et al., 1986). It has been reported that 40–80% of the flowers and pods initiated eventually abort under conventional cultivation (Wiebold et al., 1981). Abscission of flowers and young pods occurs mostly following flowering, after pollination and fertilisation have completed (Brun and Betts, 1984). Therefore, pod number is primarily determined during early stage of pod development (within 5 days after anthesis) (Dybing et al., 1986). The individual seed weight is a product of the rate and the duration of seed filling (Munier-Jolain et al., 1998), it is generally determined during seed filling after the pod number had been fixed (Westgate and Grant, 1989; Desclaux et al., 2000; Brevedan and Egli, 2003).

Both yield components (pod number and individual seed weight) are genetically determined (Tischner et al., 2003) and are subjected to environmental conditions that prevail during reproductive development (Fageria et al., 1997). Environmental stresses, such as low radiation, defoliation, and drought stress have been shown to increase the rate of pod abortion and to decrease seed size depending on the time of the stress commenced. When the stress occurs during flowering and early pod development, pod number is reduced (Egli and Yu, 1991; Westgate and Peterson, 1993; Board and Tan, 1995; Saitoh et al., 1999). When the stress occurs during seed filling, seed size is reduced while pod number remains largely unaffected (Board et al., 1994; Desclaux et al., 2000; Brevedan and Egli, 2003). As the focus of this study is on the failure of early reproductive development in soybeans, in the following, only the pod number is considered.

2.2.2. *Effect of anthesis-drought stress on pod set*

It is well recognised that drought stress during flowering and early pod development is the major cause of pod abortion in soybeans (Momen et al., 1979; Boyer, 1983; Westgate and Peterson, 1993; Saitoh et al., 1999). A brief soil water deficit during this period can

decrease pod set up to 70% (Momen et al., 1979; Andriani et al., 1991). This is because of reproductive development during this phase involves several processes that are extremely vulnerable to a change in plant water status (Saini, 1997).

According to Peterson et al. (1992) (see Fig. 6, Table 2), it is clear that during flowering and early pod growth, there is a transition from cell division in the pre-embryo and free-nuclear division in the endosperm to rapid cellularization of the endosperm and further differentiation of the embryo. Successfully passing of this transition is essential for continuous growth and setting of pods (Westgate and Peterson, 1993). The processes, such as cell division and expansion, involved in this transition are very sensitive to changes in plant water status; a high water potential of the flowers or pods is vital for setting of the pods (Westgate and Peterson, 1993). Studies have shown that soil water deficits have a direct effect on flower water status and flower function in soybean. Drought stress imposed prior to or soon after flowering can significantly decrease flower water potential (Westgate and Peterson, 1993) and impair ovary function (Kokubun et al., 2001). Similar findings have also been reported in other crops like maize where a low water potential in the style could inhibit silk elongation, arrest flower development, and disrupt ovary metabolism (Westgate and Boyer, 1985). However, to date little is known about the physiological and biochemical reasons for pod abortion in soybeans grown under drought stress during early reproductive development.

2.3. Regulation of crop reproductive abortion under anthesis-drought stress

In 2000, Saini and Westgate (2000) reviewed the progress in the research on reproductive development of grain crops as affected by drought stress. In the review, these authors concluded that in maize, wheat and rice the reproductive abortion caused by anthesis-drought stress is controlled hormonally or by the availability of carbohydrate. However, no such information is available on soybeans. Nevertheless, studies in soybean grown under well-watered conditions have indicated a role of phytohormones and carbohydrate supply in regulating pod set during early reproductive development (Brun and Betts, 1984; Dybing et al., 1986; Egli and Yu, 1991). According to these findings, it is plausible to propose that the mechanisms at work for kernel/seed abortion in drought-stressed cereals may also be the same in soybeans under drought stress. Below, a detailed review is given based on recent understanding of the physiological mechanisms regulating crop reproductive abortion under anthesis-drought stress. Comparative analyses among different crop species are also presented.

2.3.1. Carbohydrate supply – source activity

During normal ovary and seed development, sucrose is delivered via the phloem from source leaves to the developing sinks (fruits/seeds) to support the growth of these organs (Patrick, 1988). Manipulation studies involving altered radiation intensities (Schou et al., 1978; Egli and Yu, 1991; Andrade et al., 2000), elevated atmospheric CO₂ (Hardman and Brun, 1971; Mitchell et al., 1996), defoliation (Board and Tan, 1995), and supplement with various levels of sucrose (Lee et al., 1989; Aloni et al., 1997; Abdin et al., 1998) have demonstrated that there is a close linear relationship between the rate of assimilate supply and the number of fruit/seed per unit area across several crop species, including soybeans.

It is well known that the export rate of sucrose from source (leaves) to sink (fruits/seeds) organs is dependent on the current photosynthetic rate and the concentration of sucrose in the leaves (Fader and Koller, 1983; Huber et al., 1984; Grodzinski et al., 1998; Komor, 2000). The export rate of sucrose could be expressed as a positive linear function to the photosynthetic rate and the concentration of sucrose in the leaves (Fader and Koller, 1983; Huber et al., 1984). Drought stress decreases photosynthetic rate and that will apparently decrease the export rate (Huber et al., 1984). Also, drought stress may disrupt carbohydrate metabolism in the leaves. Several authors have shown that drought stress decreased sucrose concentrations in the leaves, and that was presumably due to an increased activity of acid invertase as induced by drought stress (Pelleschi et al., 1997; Kim et al., 2000). Both a low rate of photosynthesis and a low sucrose concentration in the leaves may cause a decreased rate of sucrose export to the sink organs, and thereby affect the reproductive development. As the plant growth rate is tightly correlated with the photosynthetic rate of the canopy, it is not surprising that a single linear relationship between total seed number and the plant growth rate was obtained from field peas across various cultivars, different water and temperature regimes (Guilioni et al., 2003). A fundamental role of carbohydrate supply in determining kernel set has been proposed in drought-stressed maize plants (Schussler and Westgate, 1994; 1995; Setter et al., 2001; Zinselmeier et al., 1995a,b; 1999). Under non-stress conditions, concurrent photosynthate is the predominant supplier of carbohydrates for ovary growth (Fig. 7A). At mild drought stress, concurrent photosynthate flux decreases significantly and ovary growth is largely dependent on carbohydrates supply from reserves (Fig. 7B). As the stress becomes more severe, assimilate supply from photosynthesis is totally inhibited and only a very small amount of reserve carbohydrate is available, this causes carbohydrate deprivation and eventually

leads to ovary abortion (Fig. 7C). In addition, manipulation studies showing that supplement of sucrose through stem infusion could partially rescue the loss of kernel set caused by drought stress in maize have further demonstrated the crucial role of carbohydrate supply in controlling reproductive abortion in drought-stressed crops (Schussler and Westgate, 1994; Zinselmeier et al., 1995a).

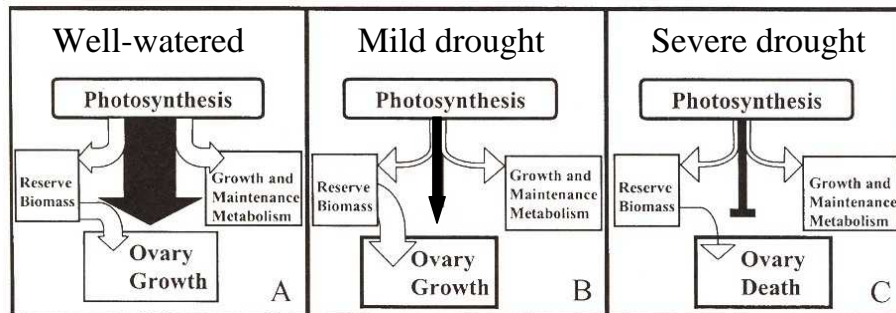


Fig. 7. Model depicting a possible relationship between concurrent photosynthate, ovary growth at well-watered and drought-stressed conditions. Modified from Gent (1994).

2.3.2. Carbohydrate utilization – sink capacity

Apart from source limitation, the capacity of the reproductive sinks to utilise the incoming assimilate (i.e. sucrose) is probably affected under drought stress and may also play a role in regulating reproductive abortion (Zinselmeier et al., 1999). It is frequently observed that sucrose concentrations in the drought-stressed ovaries are generally higher or at least similar to those of non-stressed plants (Schussler and Westgate 1991, 1995; Zinselmeier et al., 1995a). Also, the reduction in the level of starch, which is a product of sucrose metabolism in ovaries, is only partially restored by sucrose infusion (Zinselmeier et al., 1995a); and kernel abortion is not prevented completely by feeding culture medium or sucrose (Boyle et al., 1991; Zinselmeier et al., 1995a) or by increasing assimilate supply through cultural or genetic manipulations (Schussler and Westgate, 1991; Zinselmeier et al., 1995a). These studies indicate that the capacity of developing ovary to utilize the incoming sucrose is impaired by drought stress.

During early reproductive development, the young ovary receives sucrose through the phloem of the vascular system that ends in the pedicel region. This incoming sucrose is hydrolysed by acid invertases into hexoses that provide the substrate necessary for growth (Patrick, 1997). In many developing ovaries, acid invertase activity is high during the early stages of embryo and fruit development, indicating a key role of the enzyme in regulating sugar transport and utilisation during early reproductive development (Ackerson, 1985;

Weber et al., 1996; Roitsch et al., 2000; Andersen et al., 2002). Cell wall-bound acid invertase (insoluble acid invertase) is uniquely positioned to be one of the key enzymes facilitating assimilate transfer from maternal tissues to the developing embryo/endosperm, since lack of symplastic connections between these tissues necessitates an apoplastic step (Roitsch and Tanner, 1996). Vacuolar invertase (soluble acid invertase) in very young ovaries has the potential to balance metabolism, import, and osmotic contributions to growth and/or stress acclimation (Kim et al., 2000). Since the locations of cell wall-bound and vacuolar invertases in plant tissue are different, their roles in catalysing sugar metabolism may also differ. However, it has always been difficult to distinguish the specific function of the two acid invertases (Andersen et al., 2002).

Studies have consistently shown that acid invertase activity in plant reproductive structures is inhibited by drought stress (Zinselmer et al., 1995b; Dorion et al., 1996; Sheoran and Saini, 1996; Andersen et al., 2002; Qin et al., 2004). In maize ovaries, Westgate (1997) demonstrated that insoluble acid invertase activity decreased by drought stress in a manner that depended upon ovary water potential (Fig. 8). Zinselmeier et al. (1995b) reported that insoluble acid invertase activity in maize ovaries is inhibited under drought around pollination, and which parallels with the cessation of ovary growth, an accumulation of sucrose, and a decrease in the level of starch and reducing sugars. Manipulation studies with supplement of sucrose through stem injection indicated a high degree of association between acid invertase activity, ovary growth rate and kernel set in drought-stressed maize.

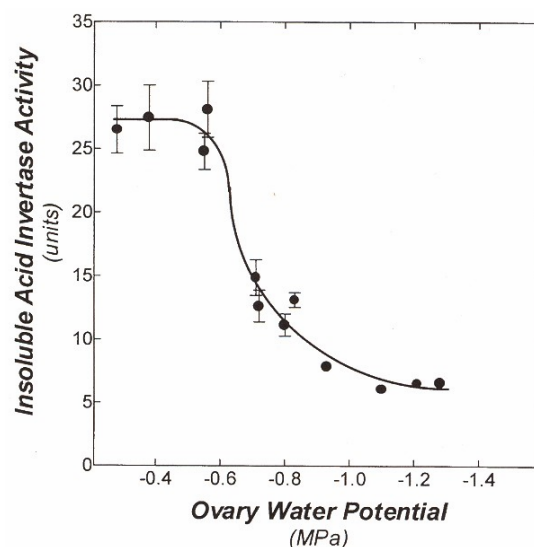


Fig. 8. Insoluble acid invertase activity in maize ovary is positively correlated with ovary water potential. Adapted from Westgate (1997).

A reduced acid invertase activity can induce a developmental arrest of reproductive tissues due to reduction of phloem unloading has recently been shown by Goetz et al. (2001) for tobacco pollen with antisense-expression of a cell wall-bound invertase. In addition, drought stress may inhibit important functions of vacuolar invertase mediated sucrose hydrolysis and osmotic potential modulation. Zinselmeier et al. (1999) observed that drought stress decreased ovary water potential and that, unless plants stems were infused with sucrose, turgor could not be maintained and decreased to a very low level. Moreover, the further metabolism of invertase-derived hexoses, mediated by hexokinase, could initiate a path of signal transduction of central importance to normal zygote development (Koch, 1996; Weschke et al., 2000; Rolland et al., 2002). For instance, a high ratio of hexoses to sucrose is occurring concurrently with a high activity of acid invertase during early embryo development of legumes, and it is thought to be crucial for cell division of young embryo and endosperm (Weber et al., 1996, 1998; Wobus and Weber, 1999). In drought-stressed maize, a low invertase activity in the young ovaries causes a decreased ratio of hexoses to sucrose, which may inhibit cell division in the developing embryo/endosperm resulting in a weak sink intensity, and may ultimately lead to abortion (Andersen et al., 2002).

Recent evidence indicates that acid invertase activity is regulated by drought stress at transcriptional level (Kim et al., 2000; Andersen et al., 2002; Trouverie et al., 2003; Qin et al., 2004). In maize, drought stress induces an increase of vacuolar invertase activity in adult leaves (Pelleschi et al., 1997; Kim et al., 2000; Trouverie et al., 2003). This induction of activity was associated with the expression of the *Ivr2* gene (encodes vacuolar invertase) but not of the *Incw2* gene (encodes cell wall-bound invertase) (Kim et al., 2000). Trouverie et al. (2003) proposed that ABA seems to be the main promoter of the *Ivr2* vacuolar invertase expression in drought-stressed adult maize leaves. These studies indicate that vacuolar invertase activity is up regulated by drought stress in source organs (e.g. adult leaf). In contrast, other studies showed that both vacuolar invertase (*Ivr2*) and cell wall-bound invertase (*Incw2*) were down regulated by drought stress in the developing maize ovaries (Andersen et al., 2002), in agreement with earlier findings of Zinselmeier et al. (1995b, 1999). The tissue/organ specific manner of the transcriptional control of vacuolar invertase expression by drought stress implies that different sets of transcription factors might be triggered in vegetative and reproduction organs (Qin et al., 2004).

2.3.3. Phytohormones

Four hormone groups, namely cytokinins (CKs), ABA, gibberellins (GA) and auxins (IAA) are important in reproductive development of crops. In normal seed development, a CKs peak shortly after anthesis is followed by a GA peak, and then by an auxin peak. ABA increases steadily during seed development (Fig. 9) (Marshner, 1995). In non-stressed, optimal environments, these hormones play important roles in co-ordinating the timing of development among ovary constituent tissues, and in regulating the overall rate of seed growth. It was generally recognised that the role for GA, IAA, and ABA lies mainly in the seed filling, whereas CKs are most important in the early stages of seed set (Hess et al., 2002). For each hormone, different patterns of endogenous concentration occur between the development of potentially strong and weak sinks during reproductive development (Wang et al., 1999; Kokubun and Honda, 2000). The roles of hormones in regulating reproductive development may be disrupted by environmental stresses such as drought and high temperature; hence they may underlie the developmental failure that is observed during stress-induced reproductive abortion (Jones and Setter, 2000). Among the four phytohormones, ABA and CKs are considered to play the most important roles in regulating early reproductive development under stress conditions and have been intensively studied. Therefore, in the following, only these two hormones are considered.

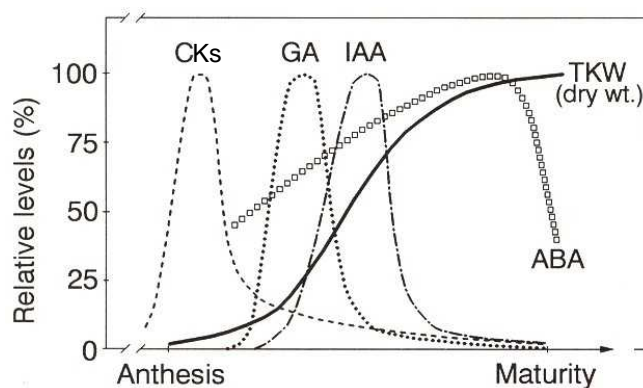


Fig. 9. Tentative patterns of phytohormone levels in cereal grains development. CKs, cytokinins; GA, gibberellins; IAA, auxins; ABA, abscisic acid; TKW, thousand kernel weight. Modified from Marshner (1995).

2.3.3.1. ABA

ABA regulates various aspects of plant growth and development, including seed maturation and dormancy, as well as adaptation to environmental stresses (Beaudoin et al.,

2000). During seed development, ABA plays several important roles, including induction of storage protein and lipid synthesis, desiccation tolerance and prevention of germination (Suzuki et al., 2000; Tian and Brown, 2000). In developing seeds, after embryonic pattern formation is complete, ABA concentrations rise while the embryo establishes dormancy and acquires storage reserves (Bonetta and McCourt, 1998). The parallel between ABA concentration and the rate of dry matter accumulation in the seeds of several grain crops indicates a role of the hormone in promoting assimilates unloading (Quebedeaux et al., 1976; Dewdney and McWha, 1979; King and Patrick, 1982; Clifford et al., 1986; Yarrow et al., 1988; Yang et al., 2003b). Stimulatory effects of ABA on in vitro sucrose uptake have also been reported (Schussler et al., 1984; Brenner et al., 1986). In soybean, early studies showed a correspondence between ABA concentration in seeds and the seed growth rate under well-watered conditions (Quebedeaux et al., 1976; Schussler et al., 1984; Ackerson, 1985; Brenner et al., 1986). These authors suggested that ABA enhances phloem unloading and may also serve to promote glucose flux from the pod wall to the developing seed. A stimulating effect of exogenous ABA on acid invertase activity in pod walls and transport of sucrose mediated by sucrose-proton symporter into the apoplast has been proposed (Tanner, 1980; Ackerson, 1985). In addition, Zhang et al. (1998) observed that in soybean seeds 30 days after anthesis, the endogenous concentration of ABA was positively correlated with the activity of acid invertase but negatively correlated with the activity of ATPase suggesting that ABA is involved in assimilate accumulation in soybean seeds. However, it is noteworthy that these effects of ABA are only significant during the linear phase of grain filling.

At early reproductive stage when cell division is active in the embryo and endosperm, endogenous ABA concentration is normally low. Elevating the level of ABA in the reproductive structures during this period may inhibit embryonic cell division and may thereby impair fruit/seed development (Wang et al., 2001). ABA concentration in crop reproductive structures increases significantly when the plants are drought-stressed during flowering (Morgan, 1980; Morgan and King, 1984; Setter et al., 2001). This increase of ABA concentration in the reproductive structures has been suggested to play a role in determining grain set in maize (Ober et al., 1991; Artlip et al., 1995) and wheat (Morgan, 1980; Westgate et al., 1996). In wheat, kernel set is negatively correlated with the endogenous ABA concentration under drought (Westgate et al., 1996); and application of ABA to the leaf sheath of well-watered plants inhibits floret development, decreases the

number of fertile florets and grain set (Morgan, 1980; Waters et al., 1984; Wang et al., 2001). Similarly, Saini (1997) showed that infusing ABA into stems of barley caused pollen abortion. Based on these studies, three mechanisms are suggested, acting individually or in combination. First, it inhibits cell division in the developing embryo/endosperm resulting in a weak sink for assimilates, causing abortion of the young ovaries (Morgan, 1980; Myers *et al.*, 1990; Mambelli and Setter 1998). Large concentrations of ABA probably inhibit cell division by depression of cell-cycle gene expression (Setter and Flannigan, 2001). Second, ABA may induce earlier closure of stomata decreasing photosynthesis hence reduce the rate of carbohydrate supply (Waters et al., 1984; Zinselmeier et al., 1999). Third, ABA may disrupt carbohydrate metabolism within the ovaries by affecting carbohydrate catalysing enzyme activity (Radley, 1979; Trouverie et al., 2003). However, the proposition that ABA induces reproductive abortion in drought-stressed crops has been obtained mostly from studies based on correlative analysis or based on the effects of exogenous ABA application on grain set of the non-stressed plants, direct evidence is still lacking. Therefore, additional studies are needed to verify the above hypotheses.

2.3.3.2. CKs

CKs are implicated in many development processes and environmental responses of plants, including leaf senescence, apical dominance, chloroplast development, anthocyanin production, and the regulation of cell division and sink/source relationship (Noodén et al., 1990; Noodén and Letham, 1993; Hutchison and Kieber, 2002). In contrast to ABA, CKs promote cell division and cell expansion and is regarded as an antagonist to ABA (Shashidhar et al. 1996, Bano et al., 1993). CKs concentrations increase after fertilisation in many species when rapid cell division is occurring (Schreiber, 1990; Jones et al., 1990; Lur and Setter, 1993). Evidence from soybean, maize, rice, barley, and wheat implicates CKs as promoters in establishing sink potential at early stages of reproductive development (Carlson et al., 1987; Schreiber, 1990; Lur and Setter, 1993; Dietrich et al., 1995). In maize, application of CKs could enhance kernel set and sometimes increase grain yield (Dietrich et al., 1995; Guo et al., 1995). In rice (*Oryza sativa*), cell division rate in the endosperm was closely correlated with the endogenous CKs (Z + ZR) concentrations and exogenous application of kinetin could increase endosperm cell number (Yang et al., 2002a, 2003a). In wheat, exogenous application of zeatin has been shown to promote floret development and significantly increase the number of fertile florets as well as grain set

(Wang et al., 2001). Studies have shown that exogenous application of CKs-like substrates, e.g. 6-benzylaminopurine (BA), to individual racemes or to the transpiration stream could prevent pod abortion in well-watered soybeans (Carlson et al., 1987; Peterson et al. 1990; Mosjidis et al., 1993; Reese et al., 1995; Nagel et al., 2001; Cho et al., 2002). Mosjidis et al. (1993) observed that BA-treated soybean ovaries had significantly more starch-filled amyloplasts and higher ^{32}P uptake rate than those without BA treatment. Application of CKs stimulated sucrose transport into cultured spikes of wheat resulting in more grain set (Borkovec and Prochazka, 1992; Lejeune et al., 1998). These lines of evidence indicate that CKs can increase sink strength by promoting cell division in the young ovaries, and this may redirect movement of assimilates into treated tissues, increase growth rates of the developing ovaries and thereby decrease the rates of abortion.

Despite such an important role of CKs in regulating early reproductive development in crop plants under non-stressed conditions, there are only a few studies, which have attempted to investigate the effect of anthesis-drought stress on endogenous concentrations of CKs in the crop reproductive structures and their possible relationships to fruit/seed abortion. In maize, Setter et al. (2001) reported an inconsistent effect of water deprivation on CKs concentration, thus at the pre-pollination stage, water deficit increased CKs concentration in apical florets, whereas at the post-pollination stage water deficit decreased CKs concentration in apical and basal pedicels. Based on these results, the authors were not able to show any relationship between kernel set and kernel CKs concentrations. Other studies have also shown contrasting effects of drought stress on CKs concentrations in the plants (Hare et al., 1997). Drought stress substantially decreased CKs concentrations in the leaves in tomato (Pillay and Beyl, 1990), rice (Bano et al., 1993; Yang et al., 2002b), and wheat (Yang et al., 2003b); but increased CK concentrations in the xylem sap in lychee trees (Stern et al., 2003).

2.3.4. Long-distance signals

2.3.4.1. Phytohormones act as long-distance signals of soil water availability

Plant roots are recognised as the source of signals that influence physiological responses of the aerial parts of a plant (Davies and Zhang, 1991). The involvement of root-originated ABA and CKs as early chemical signals in regulating shoot physiology of drought-stressed plants has been intensively studied across several species (Davies and Zhang, 1991; Bano et al., 1993; Shashidhar et al., 1996; Stoll et al., 2000; Hansen and Dörffing, 2003).

However, their roles as root-to-shoot signalling in regulation of crop reproductive development have received less attention (Mingo et al., 2003).

There is a wealth of evidence indicating that the source of stress-induced ABA that accumulates in crop reproductive structures during their period of cell division is the maternal tissues, not the finial tissues (Goldbach and Goldbach, 1977; Setter et al., 1981; Morgen and King, 1984; Ober and Setter, 1992). In *vitro* cultured maize kernels accumulate only modest amounts of ABA in response to low water potential treatments (–2.0 MPa) applied via culture media (Myers *et al.* 1992). Moreover, the increase in ABA concentration in the xylem of droughted wheat plants suggested that some of the ABA accumulated in floral organs might be derived from the roots (Munns and Sharp, 1993). A direct confirmation of above suggestions could be shown with the experiments by using a split root system, in which half the roots is drought-stressed while the remainder is kept well-watered (Dembinska et al., 1992). In this system, water uptake by the wet roots maintains the leaf water potential at the normal level, whereas the ABA produced in the dry roots is transported to the spike causing the spikelet ABA to increase to high concentrations as observed when the entire root system is stressed. Alternatively, studies in wheat using root-zone pressurization to maintain leaf water potential while roots experience soil dry-down, also support a scheme where ABA synthesis in roots is transported to shoots via xylem and is subsequently translocated into kernels via the phloem (Westgate et al., 1996).

Similarly, as the roots are the primary sites of CKs biosynthesis, xylem-borne CKs are thus probably the major source of CKs in the reproductive structures (Carlson et al., 1987; Bano et al., 1993; Yang et al., 2002a). Drought stress may decrease the delivery rate of CKs in the xylem sap (Shashidhar et al., 1996; Stoll et al., 2000; Hansen and Dörffling, 2003), reduce the amount of CKs transported from roots to the reproductive structures, and eventually leads to low levels of CKs in these tissues.

Although there is no direct evidence supporting a role of xylem sap ABA and CKs to act as early signals in regulating fruit/seed growth and development under drought stress, such effect is plausible given the significant roles of these hormones in controlling reproductive development in well-watered plants. In accordance with this, Mingo et al. (2003) suggested that root-originated chemical signals controlled fruit growth of tomatoes during partial root zone drying. However, studies of Dembinska et al. (1992) using wheat plants in a split root system indicate that soil drying–induced increase in xylem sap ABA concentrations has no

direct effect on grain set. A reduction in grain set occurred only in plants whose entire root system was stressed and leaf water potential had significantly decreased. Because roots are the primary source of CKs, the results of these experiments also may be used as an argument against a major role of CKs in regulating grain set. Consistent with this is the observation that root pressurization to restore high level of water potential in drought-stressed plants improved grain set, implying that shoot water potential is a more important determinant of grain set than root water potential (Westgate et al., 1996). Therefore, to illustrate the role of ABA and CKs as root-originated chemical signals in regulating crop reproductive development during drought stress, further investigations are required.

2.3.4.2. Sugars as long-distance signals

Recent studies indicate that the import of sugars to the reproductive structures not only serves to provide energy and storage material but also act as signals having a direct influence on gene expression, cell division, organ development (Koch, 1996; Roitsch, 1999; Smeeckens, 2000; Rolland et al., 2002; Gibson, 2004). Genes encoding acid invertase, the enzyme catalysing the first step in sucrose metabolism, are regulated in this fashion (Koch, 1996; Xu et al., 1996). The findings that one of the cell wall-bound invertase isozymes of *Chenopodium rubrum* (Roitsch et al., 1995), tomato (Godt and Roitsch, 1997), tobacco (Krausgrill et al., 1996) and *Arabidopsis* (Tymowska-Lalanne and Kreis, 1998) is induced by glucose support the significance of the metabolic regulation of the enzyme in response to sugars. It has been known that drought stress decreases photosynthesis and disrupts carbohydrate metabolism in both source and sink organs leading to changes in the concentration and flux of certain sugars, which may have a direct effect on expression of genes related to acid invertases in those organs. In maize ovaries, Andersen et al. (2002) reported that *Ivr2* acid invertase is down regulated by drought stress, and the levels of mRNA correlated negatively to sucrose levels but positively to hexose levels. However, it is noteworthy that rather than the concentrations of sugars, the flux of sugars are more important in acting as a signal in regulation of gene expression (Koch, 1996). Therefore, the above results of Andersen et al. (2002) should be interpreted very carefully that the sugar composition in the ovaries might be the effect and not the cause of a change in invertase activity. In this context, the decrease in the expression of *Ivr2* may be ascribed to a decline of incoming sucrose flux resulting from a reduced rate of photosynthesis and a low leaf sucrose concentration at low leaf water potentials (Smeeckens and Rook, 1997). That flux of sucrose is influencing gene expression of acid invertase is

supported by findings of Zinselmeier et al. (1999) and McLaughlin and Boyer (2004); these authors showed that supplement of sucrose to maize stem could partially restore the activity of acid invertase in the ovaries from drought-stressed plants.

However, studies in adult maize leaves showed opposite response of gene expression of acid invertase to drought stress (Kim et al., 2000). Recent studies of Trouverie et al. (2003) demonstrated that ABA but not sugars is seemingly the main promoter of *Ivr2* vacuolar invertase expression in drought-stressed adult maize leaves. Apparently, at a whole plant level, a down regulation of acid invertase in reproductive structures with a simultaneous up regulation of the enzyme in source organs (e.g. adult leaves) by drought stress might be of particular significance for the plant to adapt to carbohydrate deprivation under drought conditions. On one hand, high concentrations of hexoses due to greater acid invertase activity in leaves could help to maintain leaf turgor pressure, which may improve the chance of survival during prolonged drought. On the other hand, drought-induced repression of acid invertase activity in reproductive structures may limit sucrose transport and utilisation resulting in partial reproductive abortion. This may confer a critical survival advantage for few versus many seeds under drought conditions and thus secure a sufficient carbohydrate supply for maturation of a few remaining seeds (Andersen et al., 2002).

2.3.4.3. Cross-talk between phytohormone- and sugar-signalling pathways

Several studies have demonstrated that there are interactions between sugar and phytohormone signalling pathways during plant response to environmental stress (e.g. Finkelstein and Gibson, 2001). Modulation of sugar signalling responses by phytohormones or vice versa, or cross talk between the underlying signalling pathways has been determined for all of the five major phytohormones, including ABA (Kim et al., 2000; Rook et al., 2001) and CKs (Roitsch, 1999). Recent results in *Arabidopsis* with carbohydrate-insensitive mutants (*isi*, *gin*) and ABA-deficient mutants (*aba*, *abi*) have demonstrated an interaction between ABA and the sucrose signalling pathways (Rook et al., 2001, and references therein). Similarly, Pelleschi et al. (1997) observed that an accumulation of hexose in maize adult leaves is associated to an increase of xylem sap ABA concentration under water stress. Further, Trouverie et al. (2003) observed that ABA supply enhanced *Ivr2* gene expression in adult maize leaves, while sucrose supply did it less intensively and with a different time-course. However, this seems not the case in the reproductive sinks. Andersen et al. (2002) found no relationship between ovary ABA concentration and *Ivr2* gene expression in drought-stressed maize. This agrees with earlier

findings by Ober and Setter (1990) showing that drought-induced increase of ABA concentration in maize kernels has little effect on invertase activity measured *in vitro*. While studies in sorghum showed that invertase activity in the developing grain is depressed by exogenously applied ABA (Bhatia and Singh, 2002).

Interactions between CKs and sugars in the regulation of invertase activity have also been reported. An increase in acid invertase activity in response to exogenous CKs was observed in *C. rubrum* (Ehness and Roitsch, 1997) and tomato (Godt and Roitsch, 1997). The induction of invertase activity by CKs is significant in establishing sink strength by inducing cell division under a high concentration of hexoses, where a sugar signalling mechanism is involved. A well-established sink (with large number of cells) will facilitate phloem unloading and assimilate uptake. An increased rate of sugar uptake in response to CKs treatment has been demonstrated in suspension cultured cells of *C. rubrum* (Ehness and Roitsch, 1997) and cultured spikes of wheat (Wang et al., 2001). In turn, the increased sugar flux may induce the expression of genes of invertase (Godt and Roitsch, 1997), which could provide a feed forward mechanism to maintain or amplify the CKs signal.

3. A brief summary of the experimental studies in soybean

In soybean, loss of seed yield is maximal when drought stress occurs during flowering and early pod development. The yield loss is due mainly to an increased rate of pod abortion leading to a less number of pods per unit area. However, what controls pod abortion in drought-stressed soybean is still poorly understood. The aim of this thesis is to investigate the physiological reasons that are responsible for pod abortion caused by anthesis-drought stress in soybeans. The experimental studies are detailed in the succeeding four papers (I, II, III, IV), here only a short version is presented.

3.1. Materials and methods

Four pot experiments were conducted in a climate-controlled glasshouse during 2002 and 2003, where soybeans were subjected to different water regimes or hormone treatments at early reproductive development. The biophysical and biochemical items considered and the methods and instruments used to determine those items during the experiments are listed below (Table 3).

Table 3. Measurements, methods and instruments used during the experimental studies

Measured items	Methods and instruments
Leaf water potential	Pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA)
Flower and pod water potential	Psychrometers (C-52 chambers, Wescor Inc., Logan, UT, USA)
Root water potential and xylem sap collection	Pressure chamber (self-made, vol. = 10 l), xylem sap was collected by pressurising the whole root system at a pressure close to the absolute value of the leaf water potential
Gas exchange rates (photosynthesis and stomatal conductance)	LI-6200 portable photosynthesis system (LiCor Inc. Lincoln, NE, USA)
ABA concentration in xylem sap, leaf, and reproductive tissues	ELISA (Enzyme Linked ImmunoSorbent Assay)
Carbohydrate composition in leaves and reproductive tissues	HPLC (Hewlett Pacard 1100, Waldbronn, Germany)
Soluble acid invertase activity	Enzymatic-chemical methods

In addition to the above measurements, pod fresh weight and pod set percentage were also determined. It should be noted that in soybeans flowering, pod development, and rapid

seed growth might occur simultaneously. This makes it difficult to ascertain the sensitivity of a specific developmental phase to drought stress and to clarify the effect of the stress on pod set. In order to overcome this difficulty, in the experimental studies of this project we focused on the effect of drought stress on the flowers of mid-canopy main-stem racemes, as these flowers opened generally within 1-2 day around anthesis, so that their development was almost synchronous. By carefully monitoring the development of those flowers along soil drying, the effect of soil water deficits on pod growth and pod set at a certain developmental stage was examined.

3.2. Results and discussion

3.2.1. *The critical stage of pod abortion*

The results clearly showed that a period 3 to 5 days after anthesis (DAA) is the critical stage of pod abortion in soybean subjected to drought stress (**Fig. 6; Paper I**). This result is in accordance with early findings of Westgate and Peterson (1993), who reported that early pod expansion is the most sensitive stage of soybean to soil water deficits. In addition, the period also corresponds closely to the developmental stage when pod abortion starts in well-watered soybeans (Abernethy et al., 1977; Huff and Dybing, 1980; Brun and Betts, 1984; Dybing et al., 1986). Therefore, it is possible that the underlying mechanisms controlling pod abortion in well-watered soybean is simply enhanced by drought stress. During 3-5 DAA in the ovary of soybeans there is a transition from growth by cell division to growth by cell enlargement and differentiation (Peterson et al., 1992). The processes involved in this transition are very sensitive to environmental stresses. Manipulation studies in well-watered soybean plants have shown that changes in the availability of photosynthate and the level of phytohormones of the plants during this period had substantial effect on pod set (Brun and Betts, 1984; Dybing et al., 1986; Egli and Yu, 1991). Accordingly, pod abortion would be expected to increase during soil water deficit as it decreases tissue water potential and the flux of photosynthate supply from source leaves to the pods. Also, soil water deficit may change the concentration of ABA in the pods, and thereby induce pod abortion in drought-stressed soybean.

3.2.2. *Source and sink limitations and their relationships to pod set*

Limitations to crop yields are often sought in either source or sink restrictions. The source activity, which determines the availability of assimilates and the sink capacity, which determines the potential of assimilate utilisation are the two major processes involved in

determining the yield potential of a crop (Egli and Bruening, 2001). Modification of source activity of soybean during flowering and early pod expansion usually results in a corresponding change in pod number and this has been well described under optimal soil moisture conditions (Egli and Yu, 1991; Board and Tan, 1995; Andrade and Ferreiro, 1996; Board and Harville, 1998). In the present study, the results showed for the first time that drought stress disrupted carbohydrate metabolism in both leaves and floral organs resulting in both source and sink restrictions (**Paper II**). These could be clearly seen from the changes of the carbohydrates compositions and soluble invertase activity in the two organs (Fig. 10) (**Fig. 4; Paper II**). In source leaves, it was observed that drought stress decreased photosynthesis, starch and sucrose concentrations but increased hexoses (glucose + fructose) concentrations, in line with earlier findings of Huber et al. (1984) (**Fig. 4; Paper II**). The change of sugar composition in soybean leaves under drought stress might have significant impact on the whole plant physiology. For instance, accumulation of hexoses will favour leaf survival by an effect of osmotic adjustment and restrict assimilate export, which however together with the decreased sucrose and starch concentrations in the leaves indicative of a strong source limitation, whereby the export rate of sucrose to the developing sinks (pods) is reduced. A low rate of current photosynthate supply at the critical stage of pod development could be lethal for setting of pods. This was obvious the case in the present study where we found pod set to be linearly correlated with leaf photosynthetic rate when the latter reached below a threshold value (Fig. 11A). This result confirms earlier findings in well-watered soybeans grown under conditions of source limitations (Egli and Yu, 1991; Board and Tan, 1995; Andrade and Ferreiro, 1996; Board and Harville, 1998).

Drought stress also disturbed carbohydrate metabolism in the flowers and pods. In these organs, drought stress increased sucrose and hexoses concentrations but decreased starch concentration (**Fig. 4; Paper II**). Drought stress also decreased soluble invertase activity and hexoses to sucrose ratio in the pods (**Figs, 5 and 6; Paper II**). These findings indicate that the capacity of the developing pods to utilise the incoming sucrose was impaired by drought stress (**Paper II**). Similar results have also been reported for drought-stressed maize ovaries (Andersen et al., 2002) and anthers of drought-stressed rice (Sheoran and Saini, 1996), implying that a common effect of drought stress on carbohydrate metabolism within crop reproductive structures may exist.

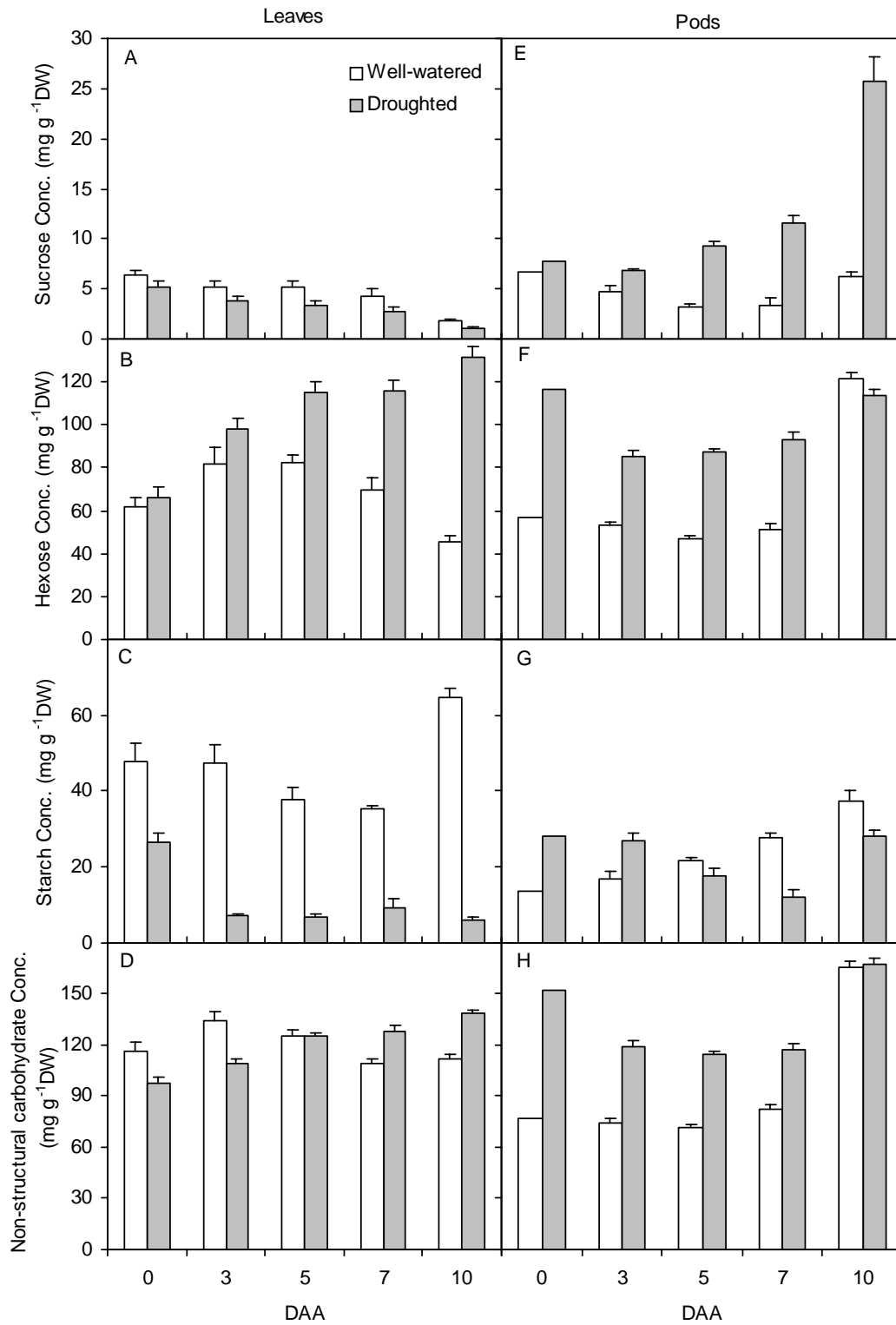
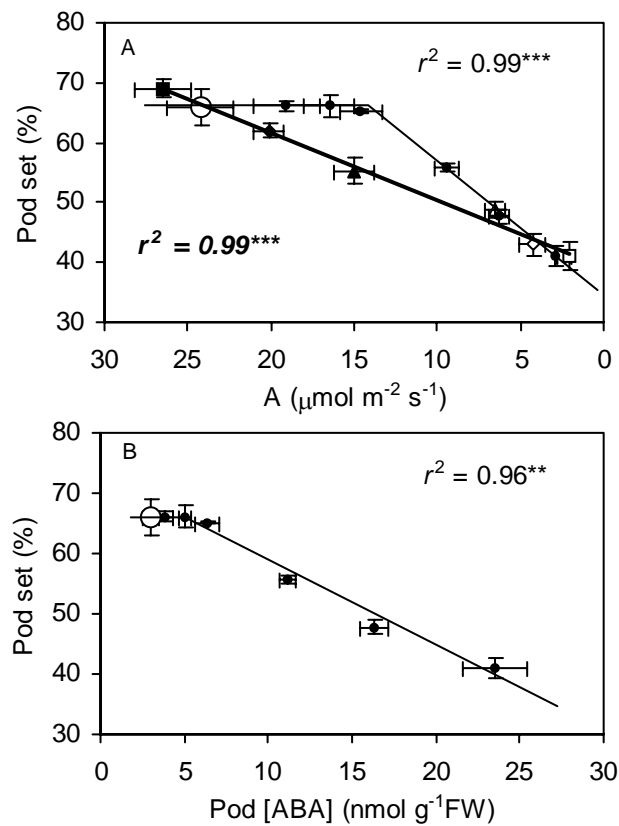


Fig. 10. Sucrose (A, E), hexose (glucose + fructose) (B, F), starch (C, G), and non-structural carbohydrate (sucrose + hexose + starch) concentrations (D, H) in soybean leaves (A, B, C, D), flowers and pods (E, F, G, H) under well-watered and drought-stressed conditions. Bars indicate SEM ($n = 4$). (Details see Fig. 4 in Paper II).

Based on this evidence, we suggest that drought-induced biochemical changes in soybean pods, i.e. reduced acid invertase activity and hence lowered hexoses to sucrose ratio at 3-5 DAA, may inhibit cell division in the ovules and pod walls resulting in a less number of cells in those tissues. A sink with fewer cells would have weak strength in attracting photosynthate from source organs, and together with a lowered availability of photosynthate in the source leaves, the amount of carbohydrate allocated into pods must have been reduced. Consistent with this, we found that the amount of non-structural carbohydrate accumulated in the pods was significantly decreased by drought stress (**Fig. 7; Paper II**).



*Fig. 11. Relationships of pod set to A (A) and to pod ABA concentration ([ABA]) (B) of soybeans subjected to different drought stresses and ABA or BA treatments. The big open cycles indicate WW plants. Bars represent SEM (n = 4). ** and *** indicate significance level at $P < 0.01$ and $P < 0.001$, respectively. (Details see Fig. 5 in Paper IV).*

3.2.3. Roles of ABA and exogenous CKs in the regulation of pod set

In the present study, our results showed that pod water potential decreased but pod turgor was maintained at high levels even in severely drought-stressed soybean (**Fig. 2; Paper I**), in accordance with early findings of Westgate and Peterson (1993) and Kokubon et al. (2001). Despite of turgor maintenance, pod growth was still decreased when $FTSW < 0.43$ indicating that low pod water potential was not the initial cause of reduction in pod growth (Figs. 12 and 13) (**Figs. 4 and 6; Paper III**). We observed that ABA concentration increased significantly in the xylem sap, leaves, and pods of drought-stressed soybean; and the decrease of pod fresh weight was closely correlated with xylem sap ABA concentration (Fig. 13) (**Fig. 6; Paper III**). Based on these findings, we proposed that root factors rather than pod water potential controls pod growth during soil drying.

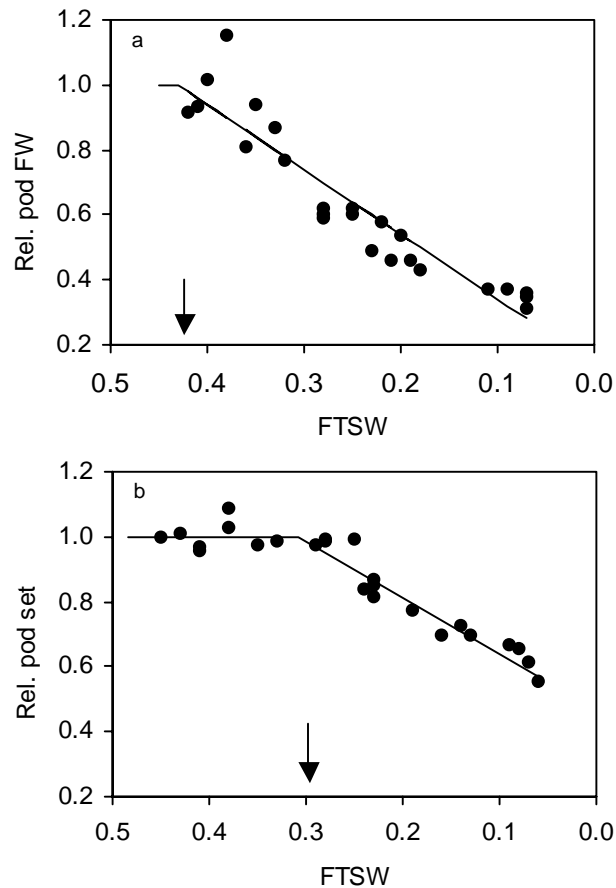


Fig. 12. Relationships between relative pod fresh weight (Rel. pod FW) (a), relative pod set (Rel. pod set) (b) and the fraction of transpirable soil water (FTSW) for soybean grown in a climate-controlled greenhouse. Relative values were the ratio of the values for the individual drought-stressed plants to the means of the well-watered plants. Arrows indicate

the threshold values of FTSW at which *Rel. pod FW* and *Rel. pod set* start to decline. (Details see Fig. 4 in Paper III).

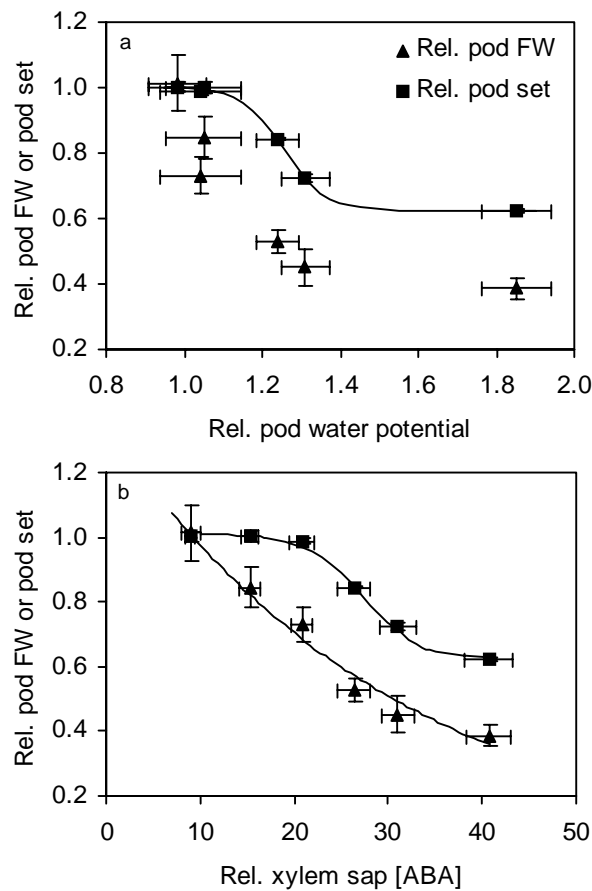


Fig. 13. Relationships of the relative pod water potential (*Rel. pod water potential*) to relative pod fresh weight (*Rel. pod FW*) and relative pod set (*Rel. pod set*) (a), the relative xylem sap ABA concentration (*Rel. xylem sap [ABA]*) to relative pod fresh weight (*Rel. pod FW*) and relative pod set (*Rel. pod set*) (b) in soybeans subjected to six levels of drought stress. Relative values were the ratio of the means of the drought-stressed plants to the means of the well-watered plants. Bars indicate SEM ($n = 4$). (Details see Fig. 6 in Paper III).

In addition, our results showed that pod set was less sensitive to soil drying than pod growth. Pod set started to decrease at FTSW = 0.31 when pod fresh weight had decreased by 30% (Figs. 4, 5, and 6; Paper III), and coincided with a decrease of pod water potential. The close association between pod set and pod water potential in drought-stressed soybean observed in this study agrees with early findings in drought-stressed wheat (Dembinska et al., 1992; Westgate et al., 1996) showing that kernel set decreases only when shoot water potential has decreased. Our results suggest that at low pod water

potentials the biochemical processes within the pods probably are disrupted, leading to pod abortion. Drought-induced disruptions in carbohydrate metabolism in the pods and their possible involvement in inducing pod abortion as reported in **Paper II** support this hypothesis.

It is well known that phytohormones play important roles in regulating crop reproductive development by affecting both sink strength and assimilate partitioning from source to sink organs (de Bruijn et al., 1993; Emery et al., 2000; Wang et al., 2001). It is also becoming clear that phytohormones may exert distinct effects at different stages during ovary development (Setter and Flannigan, 2001). During early reproductive development, it is generally found that large concentrations of ABA inhibit cell division whilst large concentrations of CKs promote cell division in the young ovary of crop plants including soybean (Emery et al., 2000; Setter and Flannigan, 2001; Yang et al., 2002a; Kokubon and Honda, 2000). In the present study, drought stress increased pod ABA concentration. Regression analysis showed that pod set decreased when pod ABA concentration had increased 1.5-fold compared to the well-watered controls. Beyond the thresholds pod set correlated negatively with pod ABA concentration (Fig. 11B). These results indicate that drought-induced changes in the endogenous concentration of ABA in the pods probably play a role in inducing reproductive abortion as had been suggested in other crops, like maize (Setter et al., 2001), wheat (Westgate et al., 1996), and rice (Yang et al., 2003a). These propositions were further confirmed by manipulation studies in which exogenous applied ABA decreased pod set in well-watered soybeans. However, in contrast to our expectation, exogenous application ABA to drought-stressed plants increased pod set (**Fig. 4; Paper IV**). The reasons for these contrasting effects of ABA on pod set in well-watered vs. drought-stressed soybeans are not clear, however, it raises the question as to whether the hormone affect pod set directly through influencing the processes within the ovary or indirectly via affecting the availability of photosynthate. Similar argument had been raised previously for seed abortion in wheat (Waters et al., 1984). Exogenous application of BA had an opposite effect on pod set to exogenous ABA in both well-watered and drought-stressed plants. This poses the same question as for ABA that what mechanisms are involved by which pod set is affected by exogenous BA. Actually, the linear relationship between pod set and photosynthetic rate obtained in the manipulation study (Fig. 11A) indicates that both direct and indirect effects of the hormones on pod set may exist. On one hand, exogenous ABA decreased photosynthetic rate and pod set in well-watered plants

whilst it increased them in drought-stressed plants; application of BA had opposite effects. In these plants pod set positively correlated with photosynthetic rate indicating that the effect of ABA and BA on pod set may be partially due to their effects on photosynthate supply (Fig. 14). On the other hand, in the well-watered plants exogenous ABA and BA affected pod set to an extent greater than that would be expected based on their effects on photosynthesis alone (Fig. 11A). This implies that direct influences of the two hormones on the metabolic processes within the pods may exist. The direct effects of the hormones within the ovary might have been on cell division and on the carbohydrate catalysing enzyme activity (e.g. acid invertase) as had been reported in maize (Radley, 1979; Mambelli and Setter, 1998; Setter and Flannigan, 2001; Trouvrie et al., 2003). However, in soybeans, these possibilities remain open for further investigations.

3.3. Conclusions

Based on the experimental results, it was concluded that:

- Early pod expansion (3-5 DAA) is the most sensitive stage for pod abortion in soybean exposed to drought stress.
- Sufficient photosynthate supply is vital for setting of pods. Drought-induced reductions of current and reserve carbohydrate supplies contribute to pod abortion during the critical, abortion-sensitive phase of pod development.
- Expansion growth of pods is more sensitive to soil water deficits than pod set. Root-originated xylem sap ABA seemingly controls pod growth, whereas low pod water potentials disrupt carbohydrate metabolism in the pods and induce pod abortion.
- ABA and BA play important roles in controlling pod set in soybeans. The two hormones exert their effect on pod set directly via affecting the pod meristem metabolic processes (e.g. cell division) or indirectly through influencing stomatal conductance and hence the rate of photosynthate supply.

3.4. Perspectives

Soybeans, as well as other grain legumes, are major source of protein and oil for human daily diet and livestock feed. Soybean plants produce more flowers than eventual pod set even under optimal soil moisture conditions. Drought stress during flowering and early pod development may increase the rate of pod abortion leading to significant yield reduction, yet the mechanisms of what controls abortion and abscission are still unknown. The results

of this thesis clearly show that drought-induced carbohydrate deprivation and increase in ABA concentrations in the pods are important components of a regulatory system by which the reproductive potential of the plant is determined. However, the cause of pod abortion is not fully understood. Future studies should focus on the following aspects:

- The molecular and genetic basis for the regulation of acid invertase activity in the pods under drought stress. This includes the roles of long-distance signals, i.e. sugar and phytohormones (ABA and CKs), in the regulation of gene expression of acid invertase. Following such lines, it might be possible to improve drought resistance of soybean through modification of the response of the genes encoding acid invertase.
- The effect of drought stress on endogenous concentration of CKs in the pods and their relations to pod set.
- The possible involvement and interaction of other phytohormones, such as ethylene, in the regulation of pod set in drought-stressed soybean.
- The potential of using exogenous ABA and BA to manipulate plant response of field-grown soybean under diverse soil moisture conditions. As our results showed that exogenous application of BA could increase pod set in well-watered soybeans, but failed to prevent pod abortion in drought-stress plants; whereas exogenous application of ABA had an opposite effect. Based on these findings, it might be possible to apply the two hormones to achieve maximize pod set at a given soil moisture condition.

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